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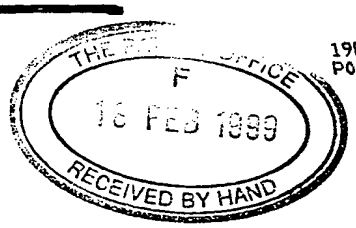

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RECEPTOR ASSAY

5. Name of your agent *(if you have one)* CRUIKSHANK & FAIRWEATHER

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Receptor Assay

The present invention relates to receptor/reporter fusion protein based assays for detecting an effect test compounds have on a particular membrane receptor, as well as to receptor/reporter fusion proteins for use in such assays and compounds identified by the assays as having interesting/useful effects.

Traditional protocols for the measurement of ligand activity at receptors such as G-protein coupled receptors (GPCRs) have relied upon a number of biochemical techniques. These include radioligand binding analysis in which the ability of a test compound to displace the binding of a known radioligand is determined, and a number of functional assays in which the ability of a test compound to activate or inhibit a specific signal transduction event is measured.

Functional assays of ligand activity at GPCRs expressed in mammalian cells include the measurement of the rate of guanine nucleotide exchange at the activated G-protein alpha sub-unit (Wise *et al.*, 1997), the measurement of the changes in the level of one of a plethora of intracellular second messenger metabolites, such as cAMP, calcium, or inositol phosphates (Guderman *et al.*, 1996), or the activation or inhibition of an ion channel (Walker and de Waard, 1998). In recent years these assays have been supplemented by the development of reporter gene systems for the study of GPCR signal transduction (Stratowa *et al.*, 1995; Alam and Cook, 1990), as well as a number of other

mammalian cell, yeast or Xenopus melanophore based assays.

The Aequorea victoria photoprotein GFP (Green Fluorescent Protein) is a 238 amino acid protein that emits green light with an emission maximum of 509nm upon fluorescent excitation at 488nm. Unlike other bioluminescent reporter proteins no additional substrates or cofactors are required for light emission (Chen *et al.*, 1995). GFP fluorescence is stable and has been measured non-invasively in living cells of many species including mammalian cells, drosophila, *C. elegans*, yeast and *E. coli*. GFP fluorescence can be detected by fluorimetry, by FACS and by microscopy. As there is no assay reagent or assay protocol the attractiveness of GFP as a reporter protein is cost, together with the speed and simplicity of the assay (Chalfie and Kain, 1998).

The availability of the cDNA sequence for GFP has resulted in the generation and characterisation of several GFP mutants with enhanced fluorescence emission. Mutation of the serine at amino acid 65 to threonine has resulted in the generation of a protein with a 6-fold increase in the intensity of fluorescence emission (Haas *et al.*, 1996). Furthermore, the presence of the Ser65Thr and the mutation of the phenylalanine residue at position 64 to leucine has resulted in a 35-fold increase in fluorescence intensity (Haas *et al.*, 1996). In addition, a number of novel mutants of GFP have also been identified with altered excitation or emission characteristics. For example mutation of the tyrosine residue at position 66 to

histidine has generated a protein with blue fluorescence emission, the so-called blue fluorescent protein (BFP) with a λ_{max} for excitation of 458 nm and for emission of 480 nm (Chalfie and Kain, 1998). These and many other variants of GFP protein are now commercially available.

GFP has been widely used in fusion proteins to assess protein trafficking, and subcellular localisation of recombinantly expressed proteins (Wang and Hazelrigg, 1994). Recently, a number of groups have described the creation and use of GPCR-GFP fusion proteins to monitor receptor internalisation and recycling following agonist treatment. For example a fusion protein between the β_2-adrenoceptor and GFP has been used to monitor receptor expression, localisation at the plasma membrane and internalisation following agonist stimulation (Barak *et al.*, 1997).

In recent years a number of studies have described the introduction of specific mutations into GPCRs that result in agonist-independent activation of a signal transduction cascade by the mutant GPCR when expressed in mammalian cells (Scheer and Cotecchia, 1997, Leurs *et al.*, 1998). This phenomena has been described as constitutive activity, and such mutant receptors termed constitutively active mutant (CAM) receptors. Such experiments have generally been considered to shed light on possible structural alterations in the GPCR which occur upon agonist-binding to result in activation of a cognate G protein and thus regulation of the activity of downstream effector enzymes.

Such strategies appear to possess validity because, in the case of the β_2-adrenoceptor for example, one of the structural modifications associated with agonist binding to the wild type GPCR is a movement of transmembrane helix 6 which can be measured by the positioning of residue Cys^{285} (Gether *et al.*, 1997a). In a CAM form of this GPCR this same Cys residue is closer to the ligand binding pocket than in the ligand-unoccupied wild type receptor (Javitch *et al.*, 1997).

Perhaps the most studied of the CAM GPCRs is a form of the human β_2-adrenoceptor in which a short segment of the C-terminal region of the third intracellular loop was replaced with the corresponding region from the α_{1B}-adrenoceptor (Samama *et al.*, 1993, Samama *et al.*, 1994).

The present invention is based in part on investigations on the possibility of developing the phenomena of ligand stabilisation of a CAM GPCR to lead to an increase in receptor number at the cell surface, as an assay system for ligand activity at such a receptor. As a model system the present applicants describe the stability and regulation, by a series of inverse agonist ligands, of a CAM β_2-adrenoceptor which has had the 27kDa GFP added in-frame at the C-terminal. The present applicants have measured ligand efficacy by determining the ability of each ligand to cause a change in the cellular distribution of the GPCR-GFP fusion protein or to cause an alteration in total cellular fluorescence. Furthermore the present applicants have examined the effect of a series of specific

agonists on the cellular distribution, and total cellular fluorescence, of cells expressing a WT β_2-adrenoceptor/GFP fusion protein as a screening system for agonist ligands at this receptor.

Thus, in a first aspect the present invention provides an assay for detecting an effect a compound has on a membrane receptor/reporter fusion protein, comprising the steps of:

a) adding the compound to a cell comprising said membrane receptor/reporter fusion protein; and

b) detecting any change of said receptor/reporter fusion protein.

Typically the assay may be used to screen compounds for their effect on particular membrane receptors. Compounds identified as having an effect on a particular membrane receptor may be useful, for example, in modulating the activity of wild type and/or mutant membrane receptors; may be used in elaborating the biological function of particular membrane receptors; and/or may be used in screens for identifying compounds that disrupt normal membrane receptor interactions, or can in themselves disrupt such interactions.

The assay is particularly suited for the detection of compounds which serve as inverse agonists, antagonists or agonists of the membrane receptor. The term inverse agonist is understood to mean a compound which when it binds to a receptor, selectively stabilises and thus enriches the proportion of a receptor in a conformation or

conformations incapable of inducing a downstream signal. Agonist is understood to mean a compound which when it binds to a receptor selectively stabilises and thus enriches the proportion of the receptor in a conformation or conformations capable of inducing a downstream signal. Antagonist is understood to mean a compound which when it binds to a receptor has no selective ability to enrich either active or inactive conformations and thus does not alter the equilibrium between them.

The term compound is understood to include chemicals as well as peptides and/or proteins.

The present invention also therefore relates to inverse agonists, antagonists or agonists of receptor proteins identified using the assays according to the present invention and to the use of such agonists, antagonists or agonists in study receptor function, or therapy.

The assay may be applied to a variety of membrane receptors, such as growth factor receptors, cytokine receptors, ion channels and integrins. The assay is however particularly suited to studying the effects of compounds on G-protein coupled receptors (GCPRs).

The term receptor as used herein is intended to encompass subtypes of the named receptors, and mutants, such as constitutively active mutants, homologs thereof, and chimeric receptors including the nucleic acid encoding such receptors. Chimeric receptors as used herein refers to receptors which may be formed comprising parts of mammalian receptors found from different sources.

Generally speaking any G protein coupled receptor, and the DNA sequences encoding such receptors may be used in assays of the present invention. Typical G protein coupled receptors are for example dopamine receptors, muscarinic cholinergic receptors, α-adrenergic receptors, β-adrenergic receptors, opiate receptors, cannabinoid receptors and serotonin receptors.

The membrane receptors mentioned herein are typically modified by the fusion of a reporter protein to the receptor. Typically nucleic acid encoding the reporter protein, such as Green Fluorescent Protein (GFP) may be fused in-frame to an end, that is the 5' or 3' end, of a gene encoding the particular receptor. In this manner, on expression of the gene, the reporter protein is functionally expressed and fused to the N-terminal or C-terminal end of the receptor. Modification of the receptor is such that the functionality of the membrane receptor remains substantially unaffected by fusion of the reporter protein to the receptor.

As mentioned previously GFP emits green light upon fluorescent excitation. Detection of this green light may be carried out for example by fluorimetry, FACS and by microscopy techniques well known to one skilled in the art. In this manner localisation and/or quantification of a membrane receptor may be determined.

The present invention in a further aspect therefore also relates to novel membrane receptor/reporter fusion proteins for use in the disclosed assays and their nucleic acid constructs, such as a constitutively active β_2-adrenoreceptor/GFP fusion protein and β_2-adrenoreceptor/GFP gene fusion. Although it might be anticipated that attachment of the GFP 27KDa polypeptide to the end of a receptor such as GPCR might significantly interfere with receptor function, it has been previously reported that GPCRs modified in this manner display unaltered pharmacology and remain able to interact with G proteins to initiate second messenger regulation. For example a receptor/reporter fusion protein may be provided in which the C-terminus of a receptor is linked directly to the N-terminus of a reporter protein. Minor modification may be carried out to the protein sequence, for example, an epitope tag may be added to the N-terminus of the receptor and/or the terminal methionine of the reporter gene removed. Many such modifications may be envisaged by the skilled addressee providing the functionality of the receptor/reporter fusion protein remains substantially uneffected.

The nucleic acid constructs of the present invention comprise nucleic acid, typically DNA, encoding the particular receptor to which is fused, in-frame, the appropriate gene encoding the reporter protein. Generally speaking the nucleic acid constructs are expressed in the cells being tested by means of an expression vector.

Typically, although not exclusively the cells are of mammalian origin and the expression vector chosen is one which is suitable for expression in the particular cell type.

An expression vector is a replicable DNA construct in which the nucleic acid is operably linked to suitable control sequences capable of effecting the expression of the membrane receptor/reporter fusion in the particular cell. Typically control sequences may include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and/or translation. Typical expression vectors may include for example plasmids, bacteriophages or viruses and such vectors may integrate into the host's genome or replicate automonously in the particular cell.

In order for the particular cell to express the receptor/reporter fusion protein the cell must be transformed by the appropriate expression vector. "Transformation", as used herein, refers to the introduction of a heterologous polynucleotide fragment into a host cell, irrespective of the method used, for example direct uptake, transfection or transduction.

The present invention therefore also relates to cells which have been transformed by nucleic acid constructs comprising receptor/reporter fusions of the present invention and express the receptor/reporter fusion protein.

In addition to the Green Fluorescent Protein (GFP) similar receptor/reporter fusion protein constructs may be made using other coloured varients of GFP such as the Blue Fluorescent Protein, the Yellow Fluorescent Protein or the Cyan Fluorescent Protein (Chalfie and Kain, 1998).

Similar receptor/reporter fusions may also be generated using other reporter proteins such as firefly (*Photinus pyralis*) luciferase (Alam and Cook, 1990). The construction of a GPCR/firefly luciferase fusion would enable the detection of compound activity using firefly luciferase activity as the read-out with detection for example in a microplate luminometer or using a CCD imaging system. Firefly luciferase assays are highly sensitive and are amenable to assay in miniturised plate formats and for detection by CCD imaging (Suto and Ignar, 1997). In addition to GFP or firefly luciferase similar receptor/reporter fusions may be generated using any reporter enzyme including *Renilla reniformis* (sea pansy) luciferase (DeWet *et al.*, 1987), secreted placental alkaline phosphatase (SEAP) (Lorenz *et al.*, 1991), β-lactamase (Moore *et al.*, 1997) and β galactosidase (Henthorn *et al.*, 1988).

Any change of said membrane receptor/reporter fusion protein as a result of adding the compound may be detected for example as a change in cellular localisation of the receptor/reporter fusion protein, or semi-quantitatively by the synthesis or degradation of said receptor/reporter fusion protein. Detection of any changes may easily be

carried out with cells placed on the surface of a microscope slide or the like. However, the assays of the present invention may conveniently be carried out on cells placed in a well of a microtitre plate or the like, such as a conventional 96-well plate.

A further modification to the assay described herein may be achieved for example by taking advantage of the route of internalisation and degredation of the PAR1 receptor. The protease activated receptor PAR1 mediates thrombin signalling. Unlike classic GPCRs such as the β_2-adrenoceptor which possess reversibly bound ligands, PAR1 (and other PAR family members), are activated following proteolytic cleavage of the N-terminus of the receptor protein by proteases such as thrombin to generate a new amino terminus that serves as a "tethered ligand", binding intramolecularly to the body of the receptor to initiate receptor signalling (Vu *et al.*, 1991a and 1991b). As with other GPCRs, following ligand activation PAR1 becomes rapidly phosphorylated and uncoupled from signalling. However, unlike classic GPCRs, PAR1 is sorted largely to the lysosomes to result in protein degredation (Trejo et al., 1998; Shapiro and Coughlin, 1998). Trafficking of activated PAR1 to the lysosomal rather then the endosomal compartment appears to be mediated entirely by the C-terminal tail of PAR1 (Trejo and Coughlin, 1999). The Substance P receptor (SPR) is activated by the peptide ligand substance P, internalised and recycled to the plasma membrane as observed for the WT β_2-adrenoceptor-GFP fusion

protein. However, exchanging the carboxyl cytoplasmic tail of the SPR for that of the PAR1 receptor resulted in the creation of an SPR/PAR1 fusion protein, which when activated by the ligand Substance P, became targeted to the lysosome for proteolytic degredation, rather than to the endosome for recycling to the plasma membrane (Trejo and Coughlin, 1999).

Thus it may be expected that a fusion protein comprising the substance P receptor, the β_2-adrenoceptor or any other GPCR, in which the cytoplasmic C-terminal tail of the receptor was replaced with the cytoplasmic C-terminal tail of the PAR1 receptor, would be internalised into lysosomes following agonist treatment to result in the degredation of the receptor protein. If this fusion protein also contained GFP, or any other reporter protein, fused in-frame onto the carboxyl terminus of the PAR1 receptor cytoplasmic C-terminal tail then agonist treatment would result in a loss of reporter signal as a result of lysosomal degredation of receptor protein. To assay for compounds with antagonist or inverse agonist activity cells would be pretreated with antagonist for a period of time prior to the addition of agonist. The antagonist would prevent agonist binding to the receptor, and so prevent agonist mediated degredation of receptor protein. Using GFP as the reporter protein this may be detected either by confocal microscopy of individual or groups of cells, or in microplate formats using a appropriate microplate fluorimeter. Using firefly luciferase as the reporter

protein receptor changes could be assessed following the assay of firefly luciferase activity using either microplate luminometry or by CCD imaging.

The present invention will now be further described by way of example only, with reference to the following figures which show:

Figure 1 shows plasma membrane location of WT β_2-adrenoceptor-GFP and internalisation in response to isoprenaline. The WT β_2-adrenoceptor-GFP was expressed stably in HEK293 cells and individual clones isolated. A patch of cells were imaged in the confocal microscope in the absence of agonist (A) and following addition of 10μM isoprenaline for 5 (B), 10 (C) and 30 (D) min.

Figure 2 shows recycling of WT β_2-adrenoceptor-GFP to plasma membrane following addition of alprenolol. A patch of HEK 293 cells stably expressing the WT β_2-adrenoceptor-GFP fusion protein were imaged in the confocal microscope in the absence of agonist (A) or following addition of isoprenaline (10μM) for 30 min (B). Following washing to remove isoprenaline, alprenolol (10μM) was added for 30 (C) or 40 (D) min.

Figure 3 shows expression of CAM β_2-adrenoceptor-GFP and upregulation by betaxolol. A CAM β_2-adrenoceptor-GFP construct was expressed stably in HEK293 cells and individual clones isolated. (a) Cells of a single clone were grown on glass coverslips in the absence (Upper panel) or presence (Lower panel) or betaxolol (10μM) for 24hr. These cells were then visualised. (b) Cells of this clone which were untreated or treated with betaxolol (10μM) and then washed were used to measure the specific binding of [^{3}H]DHA in intact cells ([^{3}H]DHA is a lipophillic antagonist which crosses the plasma membrane and thus provides a measure of total cell levels of β_2-adrenoceptor binding sites).

Figure 4 shows upregulation of CAM β_2-adrenoceptor-GFP by other β-adrenoceptor ligands. HEK 293 cells stably expressing the CAM β2-adrenoceptor-GFP expressing cells of Figure 3 were exposed to no ligand (A), carvedilol (B), Labetolol (C) or ICI118551 (D) (each at 1μM) for 24h. The cells were then imaged in the confocal microscope.

Figure 5 shows upregulation of CAM β_2-adrenoceptor-GFP but not WT β_2-adrenoceptor-GFP by betaxolol. Membrane fractions were prepared from HEK 293 cells stably expressing either the CAM β_2-adrenoceptor-GFP or the WT β_2-adrenoceptor-GFP fusion protein which had been maintained for 24 hours in the absence or presence of betaxolol (10μM) and subjected to SDS-PAGE. Following transfer to nitrocellulose, the

samples were immunoblotted using an polyclonal anti-GFP antibody to assess the level of fusion protein in these membranes.

Figure 6 shows internalisation of upregulated CAM β_2-adrenoceptor-GFP by isoprenaline (a) CAM β_2-adrenoceptor-GFP expressing cells were untreated (A) or exposed to betaxolol (10μM, 24h) (B-D). Following betaxolol treatment the cells were washed and isoprenaline (10μM) added for 0 (B), 10 (C) or 30 (D) min. (b) Cells as in Figure 6a were untreated, exposed to betaxolol (10μM, 24h) or exposed to betaxolol followed by further exposure to isoprenaline for 30min. Intact cells were then used to measure the specific binding of [^{3}H]CGP12177 ([^{3}H]CGP12177 is a hydrophillic ligand which does not penetrate the plasma membrane and in these conditions records only cell surface receptors).

Figure 7a shows the effect of various inverse agonists/antagonists on the level of fluorescence in CAM β_2-adrenoceptor-GFP determined by microtitre plate fluorimetery. Changes in fluorescence were measured on Spectrofluor Plus fluorimeter using cells plated in a 96 well plate. The graph shows the concentration responses to isoprenaline, betaxolol, alprenalol or sotalol after 22 h drug contact. Values are the mean percentages of basal of at least 3 experiments performed in duplicate ± SEM.

Figure 7b shows the concentration-dependence of the upregulation of CAM β_2-adrenoceptor-GFP by betaxolol determined by microtitre plate fluorimetry. Changes in fluorescence were measured on Spectrofluor Plus fluorimeter using cells plated in a 96 well plate. The graph shows a dose response curve to betaxolol at time 0 h (•) and after 22 h (▲). Values are the mean percentages of basal of 6 experiments performed in duplicate ±SEM.

Figure 7c shows the concentration dependence of the downregulation of β_2-adrenoceptor-GFP by Isoprenaline determined by microtitre plate fluorimetry. The graph shows a dose response curve to isoprenaline at time 0 h (•) and after 22 h (▲). Values are the mean percentages of basal of 6 experiments performed in duplicate ±SEM.

Figure 8 shows the concentration dependence of the upregulation by of CAM β_2-adrenoceptor-GFP by alprenolol. binding studies: CAM β_2-adrenoceptor-GFP expressing cells were untreated or exposed to varying concentrations of alprenolol for 24h. They were subsequently washed and intact cell specific binding of single concentrations of either [^{3}H]DHA or [^{3}H]CGP12177 measured to ascertain levels of total cell receptor and cell surface receptor respectively.

Materials and Methods

[^{3}H]DHA (64 Ci/mmol) and [^{3}H]CGP-12177 (44 Ci/mmol) were purchased from (Amersham, UK). [^{3}H]adenine and [^{3}H]cAMP were purchased from Amersham International, Amersham, U.K. All reagents for cell culture were purchased from Life Technologies (Paisley, Strathclyde, U.K.). Receptor ligands were purchased RBI. All other reagents were purchased from Sigma or Fisons and were of the highest purity available.

Construction of GFP tagged forms of the β_2-adrenoceptor

Human wild type β_2- AR in pcDNA3 (MacEwan & Milligan 1996a) was amplified by PCR using a *Hind III*-FLAG forward primer, 5' AAAAAA AAGCTT GCCACC ATG GAC TAC AAG GAC GAC GAT GAT AAG GGG CAA CCC GGG AAC GGC 3', and a *Bam HI* reverse primer, 5' AAAAA GGATCC TCC CGC CAG CAG TGA GTC ATT TGT A 3'. This removed the stop codon and the initiating methionine (start codon) of β_2-WT-AR, with an initiator ATG being present in the N-terminally added FLAG™ epitope tag (ATG GAC TAC AAG GAC GAC GAT GAT AAG). The PCR product was digested with *Hind III* and *Bam HI* and the resulting fragment ligated into pcDNA3 to generate a wild type B_2-AR/GFP construct. Sequence encoding amino acids 172-291 of WT B_2 receptor were restricted this construct using *Kpn I/Hpa I* and replaced by the equivalent region of the CAM β_2- AR (Samama *et al.*, 1993, 1994). A modified form of GFP (Zernicka-Goetz *et al.*, 1997) was also amplified by PCR using a *Bam HI* forward primer, 5' AAAAA GGATCC AGT AAA GGA GAA GAA CTT

TTC 3', and an *Xba I* reverse primer,

5' TGCTCTAGATTATTTGTATAGTTCATCCATGCC 3'. This removed the initiating methionine of GFP and the resulting PCR product was digested and linked in frame to generate the CAM β_2-AR-GFP construct

Transient and stable transfection of HEK293 cells

HEK293 cells were maintained in Minimum Essential Medium (MEM, Sigma) supplemented with 0.292 g/L L-glutamine, and 10% newborn calf serum at 37°C. Cells were grown to 60-80% confluence prior to transient transfection. Transfection was performed using LipofectAMINE reagent (Life Technology, Inc.) according to manufacturers' instructions. To generate stable cell lines, two days after transfection cells were seeded/diluted and maintained in MEM medium supplemented with 1mg/ml Geneticin (Life Technology, Inc.). Medium was replaced every 3 days with MEM medium containing 1mg/ml Geneticin. Clonal expression was initially examined by fluorescence microscopy for the GFP containing clones. Selected clones expressing GFP and non-GFP tagged forms of the receptors were expanded and [^{3}H] ligand binding studies performed to assess the level of receptor expression.

Confocal laser scanning microscopy

Cells were observed using a laser scanning confocal microscope (Zeiss Axiovert 100) using a Zeiss Plan-Apo 63 x 1.40 NA oil immersion objective, pinhole of 35, and electronic zoom 1 or 3. The GFP was exited using a 488 nm

argon/krypton laser and detected with 515-540 nm band pass filter. The images were manipulated with Zeiss LSM or MetaMorph software. Two different protocols for preparation of cells were used. When examining the time course of internalisation and recycling live cells were used. Cells were grown on glass coverslips and mounted on the imaging chamber. Cells were maintained in KRH buffer (see below) and temperature was maintained at 37°C. In other studies fixed cells were used. Cells on glass coverslips were washed with PBS and fixed for 20 min at room temperature using 4% paraformaldehyde in PBS/5% sucrose pH 7.2. After one wash with PBS coverslips were mounted on microscope slides with 40% glycerol in PBS.

[^{3}H]ligand binding studies

CAM β^2-AR-GFP cells were grown in 6 cm dishes and treated with or without 10μM betaxolol or various concentrations of alprenlol for 24 h. In some cases betaxolol treated cells were subsequently exposed to 10μM isoprenaline for 30 min. After treatment the cells were washed 3 times with ice cold phosphate- buffered saline (PBS; 2.7mM KCl, 137mM NaCl, 1.5mM KH_2PO_4, 8mM Na_2HPO_4, pH 7.4). Cells were then detached from plates with PBS/0.5mM EDTA pelleted and resuspended in ice cold Krebs-Ringer-Hepes buffer (KRH; 130mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 1.2mM $CaCl_2$, 20mM HEPES, 1.2mM Na_2PO_4, 10mM glucose, 0.1% BSA; pH 7.4) buffer. After counting the cells in a hemocytometer approximately 100, 000 cells were added to each assay tube.

For binding studies a single concentration of [^{3}H] DHA (2nM) or [^{3}H] CGP-12771 (10nM) was used to measure total cell receptor and cell surface receptor respectively. Parallel studies with 10μM propranolol allowed assessment of non-specific binding. [^{3}H] DHA binding assays were performed at 30°C for 45 min and [^{3}H] CGP-12771 binding at 14°C for 2.5 hours in KRH buffer. All experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes with ice-cold TE (75mM Tris, 1mM EDTA; pH 7.4) buffer.

Intact cell adenylyl cyclase activity measurements

Were performed essentially as described by Wong (1994) and Mercouris *et al.* (1997). Cells were split into wells of a 12-well plate and the cells were allowed to reattach. Cells were then incubated in medium containing [^{3}H]adenine (1.5 μCi/well) for 16-24 h. The generation of [^{3}H]cAMP in response to treatment of the cells with various ligands and other reagents was then assessed. Results are presented as the ratio of levels of [^{3}H]cAMP to total [^{3}H]adenine nucleotides (x 1000).

Immunoblotting studies

Electrophoresis and Immunoblot Analysis

A borate-based electrophoretic buffer system[Poduslo, J.F. (1981) Anal. Biochem. 114, 131-139] was employed with some modifications. Briefly, the resolving polyacrylamide gel was made of 10% acrylamide, 0.0625% bisacrylamide, 0.1 M

Tris (pH 8.5), 0.1 M boric acid, 0.0025 M EDTA, 0.1% SDS, 0.005% TEMED and 0.1% ammonium persulfate. The stacking gel was of the same composition except that it contained 4% acrylamide. The borate electrophoresis running buffer was composed of 0.1 M Tris, 0.1 M boric acid, 0.0025 M EDTA and 0.1% SDS (pH 8.5). Standard and borate electrophoresis were run for 1 h at 200 V and 150 V, respectively using a Mini Protean II gel kit (BIO-RAD, Hamel Hempstead, U.K.). After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose. The membrane was blocked for 1 h in 3% fat-free milk in PBS-T buffer (PBS containing 0.1% Tween 20). After a brief wash in PBS-T buffer, the membrane was incubated overnight at 4°C with an appropriate primary antibody diluted in PBS-T buffer containing 1% fat-free milk. A GFP polyclonal antibody (Clontech Laboratories, U.K.) was used for the detection of the constructs. The primary antibody was then removed and the blot washed extensively in PBS-T buffer. Subsequent incubation with secondary antibody (donkey anti-rabbit IgG conjugated with horseradish peroxidase, Scottish Antibody Production Unit, Carluke Scotland) proceeded for 2 h at room temperature and after extensive washing in PBS-T buffer the blot was visualized by enhanced chemiluminescence ECL (Amersham). Quantitative analysis of specific bands was performed by scanning with an imaging densitometer GS-670 (BIORAD).

Studies in microtitre plates

Cells were seeded into black costar view plates the day before the experiment. On the day of the experiment the media was removed from the cells and drug added to the well in a final volume of 100 μl. The experiment was preformed in phenol red free F12 media containing 10% FCS. A Spectrafluor Plus fluorimeter was used to read the plates reading from the bottom at a gain of 100. A blank plate was initially read on the flourimeter and then the plates of cells were read at time 0 and after 22hrs incubation at 37°C with drug. Results were calculated by subtracting the blank plate from the fluorescence values obtained to control for plate autofluorescence.

Example 1 Construction of GPCR/GFP fusion protein

A PCR based strategy was used to link a cDNA encoding a form of GFP with enhanced autofluorescence properties (Zernicka-Goetz *et al.*, 1997) in-frame with cDNAs encoding both the wild type β_2-adrenoceptor and a constitutively active mutant form of this GPCR, produced by replacement of a small segment of the distal end of the third intracellular loop with the equivalent segment of the hamster α_{1B}-adrenoceptor. These were anticipated to encode single open reading frames in which the C-terminus of the GPCR was linked directly to the N-terminus of GFP. Following transient transfection of these constructs and visualisation on a fluoresecence microscope to confirm successful expression and autofluorescence, both of these

constructs and the equivalent non-GFP tagged forms of the GPCRs were expressed stably in HEK293 cells. Individual clones were identified based on a combination of appropriate autofluorescence and specific binding of the β-adrenoceptor antagonist [^{3}H]dihydroalprenolol ([^{3}H] DHA) and subsequently expanded. In clones expressing the wild type β_2-adrenoceptor-GFP construct, confocal microscopy performed on intact cells grown on a glass cover slip demonstrated the bulk of the GFP-derived autofluorescence to be plasma membrane delineated (Figure 1). Addition of the β-adrenoceptor agonist isoprenaline (10^{-5} M) resulted in a time-dependent internalisation of the construct into discrete intracellular vesicles (Figure 1) as has previously been reported for such a construct (Barak *et al.*, 1997, Kallal *et al.*, 1998). The wild type β_2-adrenoceptor-GFP construct internalised following 30 min treatment with isoprenaline could be recycled to the plasma membrane following removal of isoprenaline and its replacement by the β-adrenoceptor antagonist alprenolol (10^{-5}M) (Figure 2) which did not itself promote internalisation.

Although clones expressing the CAM β_2-adrenoceptor-GFP construct were also isolated these did not display the same level of GFP autofluoresence as the clones expressing the WT β_2-adrenoceptor-GFP construct. Such observations were consistent with routinely lower levels of steady state expression of the CAM β_2-adrenoceptor-GFP construct. This was confirmed by the lower levels of [^{3}H]DHA specific

binding to membrane fractions isolated from these cells compared to clones expressing the wild type β_2-adrenoceptor-GFP construct. Furthermore, although clear plasma membrane-localised CAM β_2-adrenoceptor-GFP could be observed there appeared to be a greater fraction of the GFP autofluoresence located intracellularly than for the WT β_2-adrenoceptor-GFP (Figure 3a).

Example 2 Ligand binding to GPCR/GFP fusion protein

The present applicants have previously postulated that sustained treatment of NG108-15 cells stably expressing the CAM β_2-adrenoceptor with the inverse agonist betaxolol can cause an increase in steady state levels of this GPCR. When cells expressing the CAM β_2-adrenoceptor-GFP construct were treated with betaxolol (24h, 10^{-5} M) and then visualised by confocal microscopy a marked increase in both plasma membrane delineated and intracellular fluoresence was observed (Figure 3a). Washing of the cells followed by an intact cell ligand binding experiment with [^{3}H]DHA confirmed upregulation of CAM β_2-adrenoceptor-GFP in response to betaxolol (Figure 3b). Upregulation of fluoresence was also observed by treatment of the cells with a range of β_2-adrenoceptor inverse agonist/antagonists including ICI118551, labetolol, carvedilol, alprenolol and dihydroalprenolol (all at 10^{-5} M) (Figure 4). However, pharmacological selectivity of this effect was preserved as it was not recorded by treatment with the α_1-adrenoceptor antagonist prazosin or the α_2-adrenoceptor antagonist

yohimbine (data not shown).

Sustained treatment of cells expressing wild type β_2-adrenoceptor-GFP with betaxolol or the other ligands described above failed to result in a significant upregulation of the construct as fluorescence intensity and distribution pattern was little modified by the drug treatments (data not shown). Upregulation of CAM β_2-adrenoceptor-GFP by betaxolol treatment could also be monitored in immunoblot experiments to confirm the effects seen by confocal microscopy. Membranes isolated from either wild type β_2-adrenoceptor-GFP or the CAM β_2-adrenoceptor-GFP expressing cells following maintenance in the presence or absence of betaxolol (10^{-5} M) for 24h were resolved by SDS-PAGE and the GPCR constructs detected by immunoblotting with an anti-GFP antibody. Clear upregulation of CAM β_2-adrenoceptor-GFP but not wild type β_2-adrenoceptor-GFP was observed (Figure 5).

Following betaxolol-induced upregulation of CAM β_2-adrenoceptor-GFP removal of this ligand, and its replacement by isoprenaline (10^{-5} M), resulted in a rapid internalisation of the construct into intracellular punctate vesicles in a manner which was indistinguishable from that recorded above for wild type β_2-adrenoceptor-GFP (Figure 6a). [^{3}H]CGP-12177 is a hydrophillic β_2-adrenoceptor antagonist which is unable to cross the plasma membrane. Therefore in intact cell specific binding experiments it identifies only the cell surface population of the β_2-adrenoceptor. Such intact cell binding studies

were performed on cells expressing CAM β_2-adrenoceptor-GFP, cells which had been pre-treated with betaxolol (24h, 10^{-5} M), and such cells after replacement of betaxolol with isoprenaline (10^{-5} M) for 30 min. These studies demonstrated that cell surface upregulated CAM β_2-adrenoceptor-GFP was largely internalised by agonist treatment (Figure 6b).

Upregulation of CAM β_2-adrenoceptor-GFP by sustained treatment with betaxolol and β-adrenoceptor antagonist/inverse agonist ligands could be detected and directly quantitated in a Spectrofluor Plus fluorimeter following seeding of cells into a 96 well microtitre plate. This allowed analysis of the concentration-dependence of CAM β_2-adrenoceptor-GFP upregulation with various inverse agoinst/antagonists after 22 h incubation with the compounds (Figure 7a). Betaxolol gave the clearest response (Figure 7b) producing an upregulation of the construct with an EC_{50} of 168(47-600)nM, a value in good accordance with the measured K_i of betaxolol to bind to this GPCR construct (MacEwan & Milligan 1996a). Treatment of the WT β_2-adrenoceptor-GFP fusion with betaxalol did not result in any change in cellular fluorescence following either 1 h or 22 h of drug incubation. However, incubation of such cells with the agonist ligand isoprenaline for 22 h resulted in a marked reduction of cellular fluorescence which upon quantification in the Spectrofluor Plus fluorimeter allowed an analysis of the concentration dependence of isoprenaline mediated changes in cellular

fluorescence. Isoprenaline caused a decrease in cellular fluorescence an IC50 of 13(2.5-70) nM (Figure 7c). This contrasts with a reported EC_{50} of 5nM for the stimulation of cAMP by this drug at this receptor.

In summary this example shows that inverse agonist or neutral antagonist treatment of cells expressing a CAM β_2-adrenoceptor-GFP fusion construct results in an increase in membrane fluorescence as detected by confocal microscopy, and an increase in total cellular fluorescence as measured by microplate fluorimetry. The concentration dependence of these effects agrees with data obtained from traditional pharmacological studies thus validating the use of this approach for the characterisation of compounds which effect receptor function. The ability of inverse agonist or antagonist ligands to cause an increase in cellular fluorescence from cells expressing a CAM GPCR-GFP fusion allows for the provision of a microplate based fluorescence assay for new compounds with similar activity.

Agonist treatment of cells expressing the WT β_2-adrenoceptor-GFP fusion was observed to result in a decrease in membrane associated fluorescence and an increase in fluorescence in intracellular vesicles which by co-immunolocalisation studies with an anti-transferrin antiserum are shown to be endosomes. The decrease in fluorescence observed by micrioplate fluorimetry following internalisation of the fusion protein may be due in part to receptor degradation but may also be due to a fluorescence quenching event as a consequence of receptor concentration

within the acidic environment of the endosome compartment. However, this decrease in fluorescence caused by agonist ligands such as isoprenaline is concentration dependent and the half maximal drug concentrations required to cause this effect is in agreement with the values obtained in traditional second messenger analysis studies.

Thus the example discloses a novel screening system for compounds with either agonist, neutral antagonist or inverse agonist activity at the β_2-adrenoceptor in which compound activity results in a change in the fluorescence characteristics of cells expressing a β_2-adrenoceptor-GFP fusion protein. The change in the fluorescence characteristics can be measured by either a change in cellular localisation using the confocal microscope, or by a change in total cellular fluorescence as measured in a 96-place fluorimeter. Using confocal microscopy as the detection system, agonist ligand would cause an increase in cell surface fluorescence of the CAM GPCR/GFP fusion protein while antagonist/inverse agonist ligands cause an increase in internalisation of a WT GPCR/GFP fusion protein. Using microplate fluorimetry as the detection system inverse agonist or antagonist ligands would cause an increase in total cellular fluorescence in cells expressing the CAM GPCR/GFP fusion protein while agonist ligands would cause a decrease in total cellular fluorescence in cells expressing a WT GPCR/GFP fusion protein.

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FIGURE 1

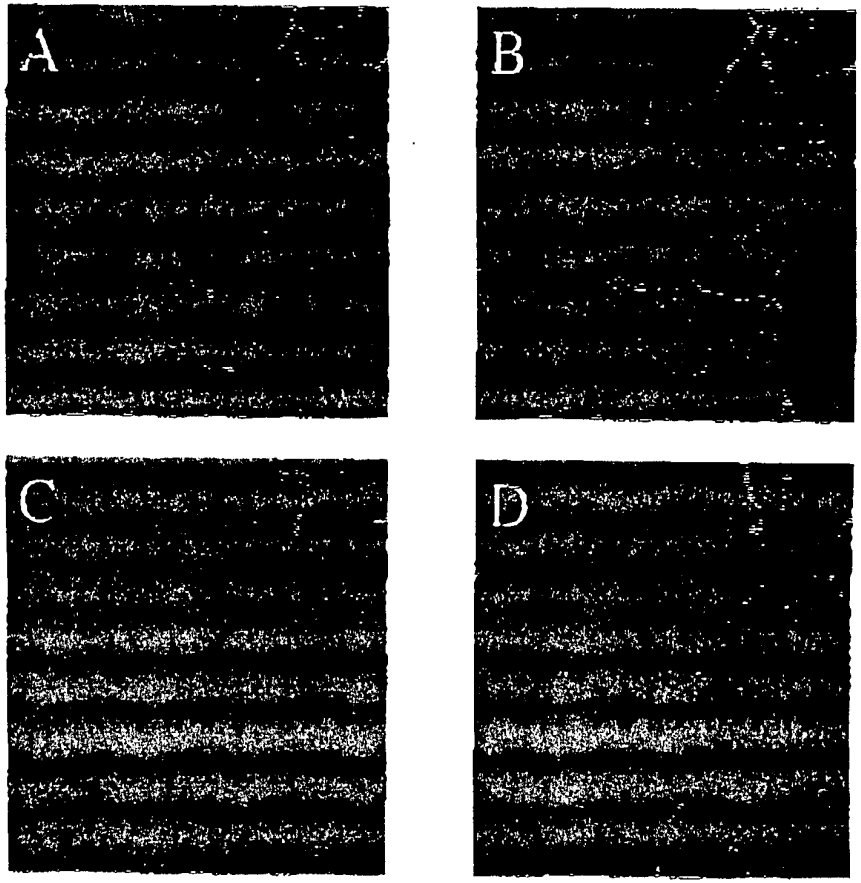


FIGURE 2

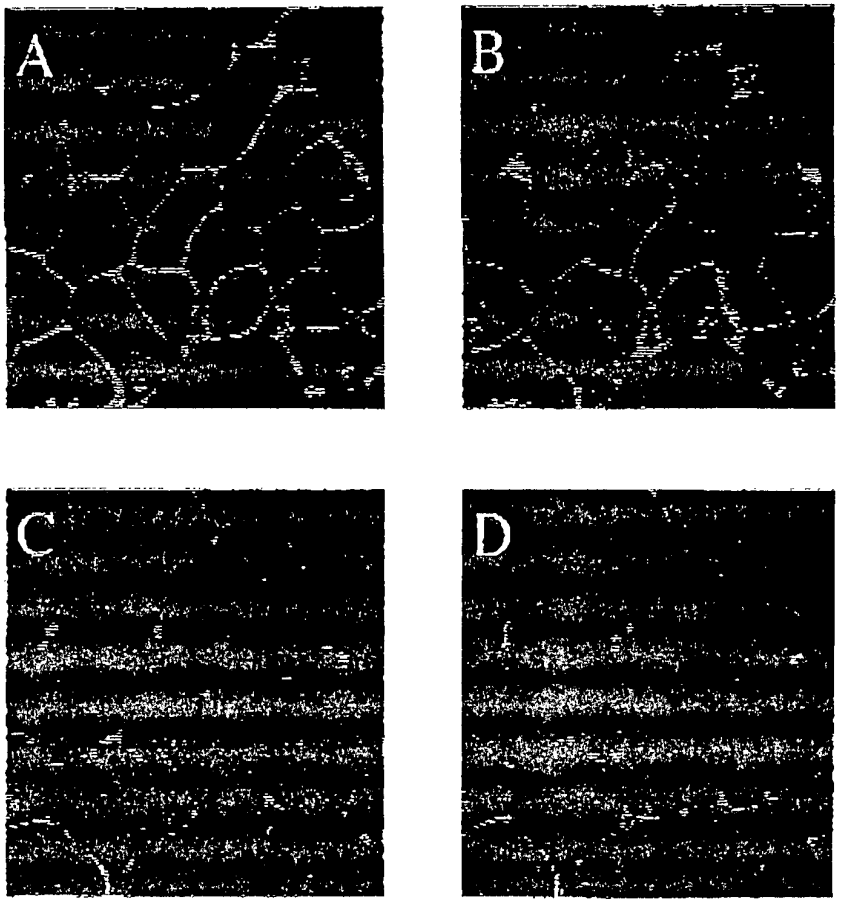
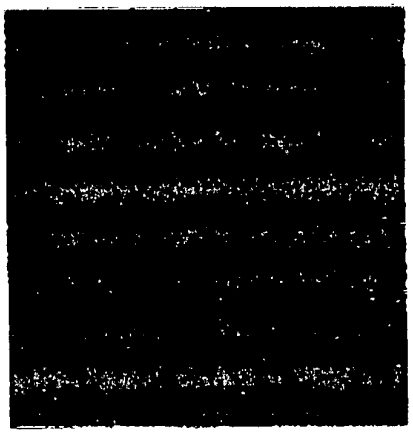


FIGURE 3



(-) Betaxolol

(+) Betaxolol

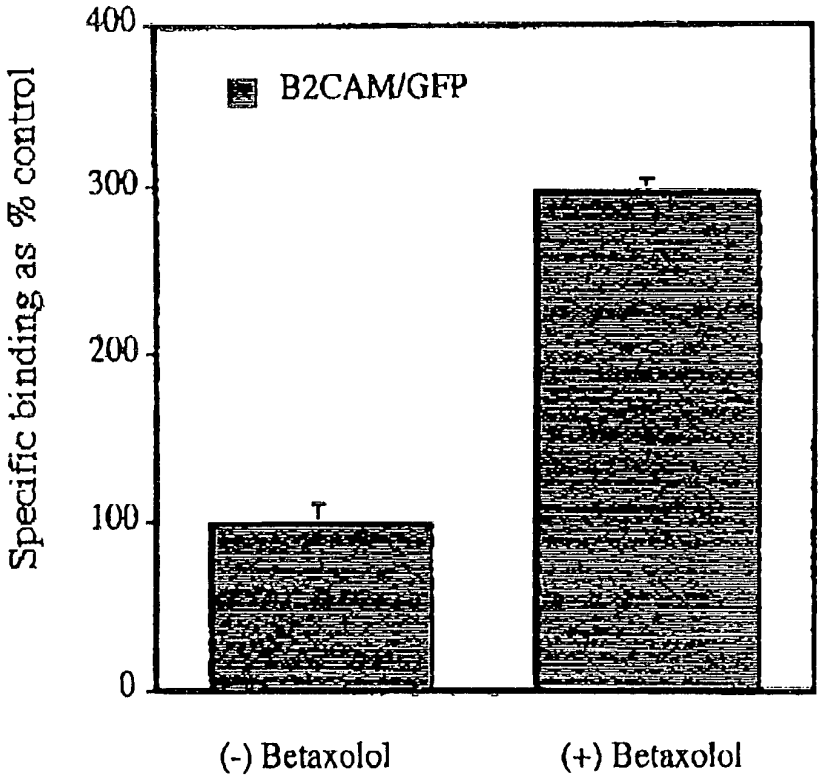


FIGURE 4

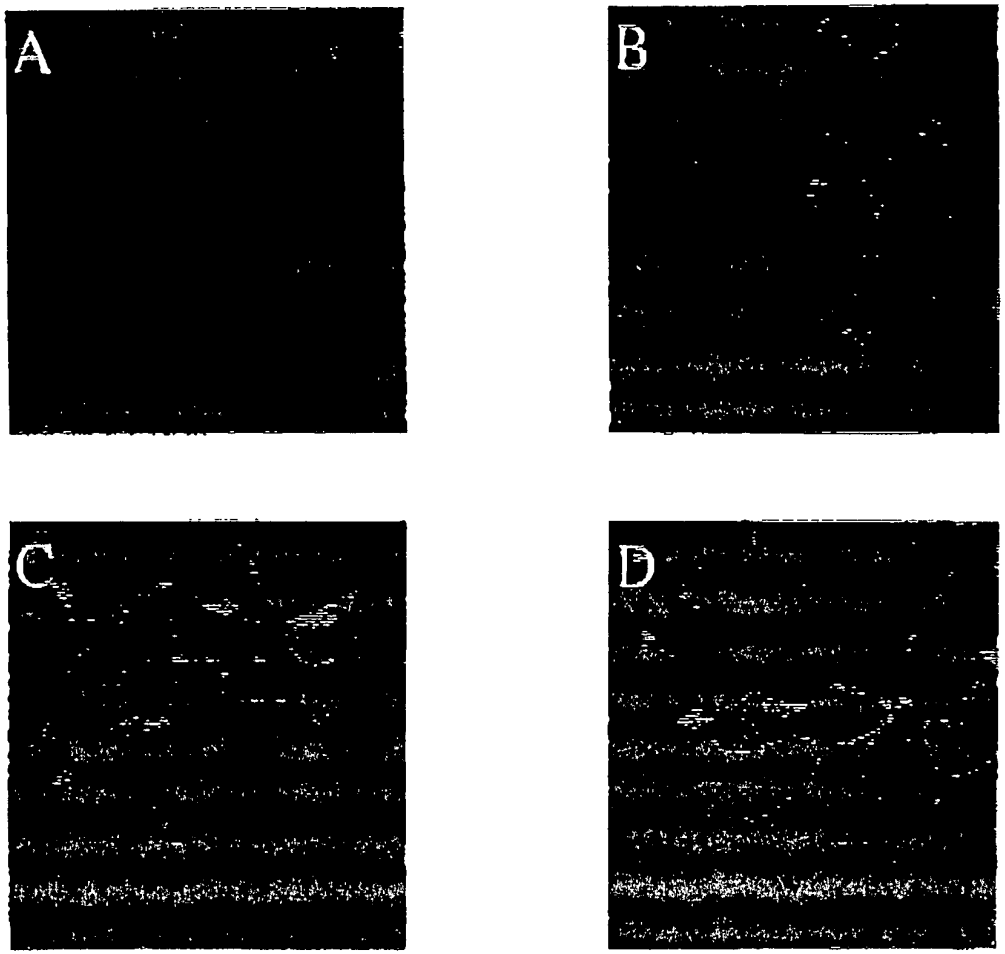


FIGURE 5

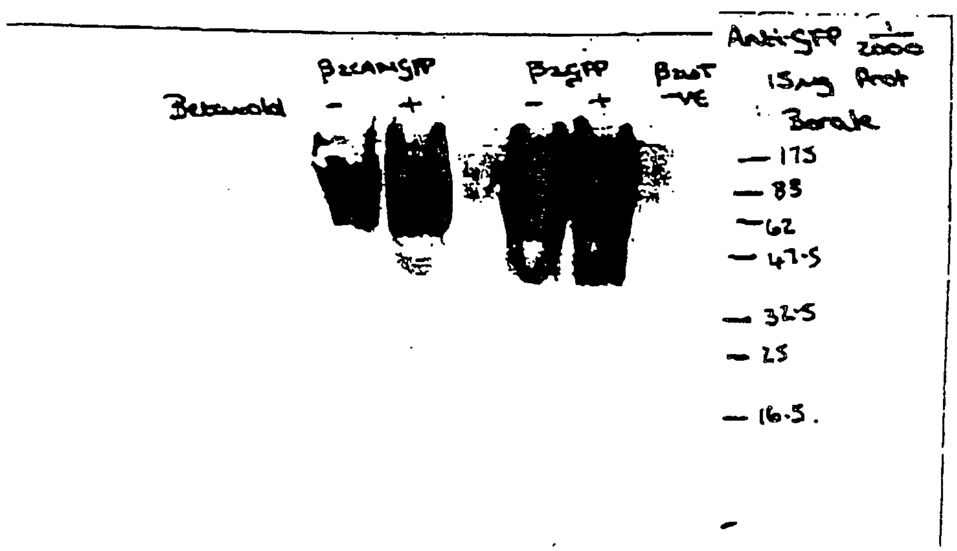
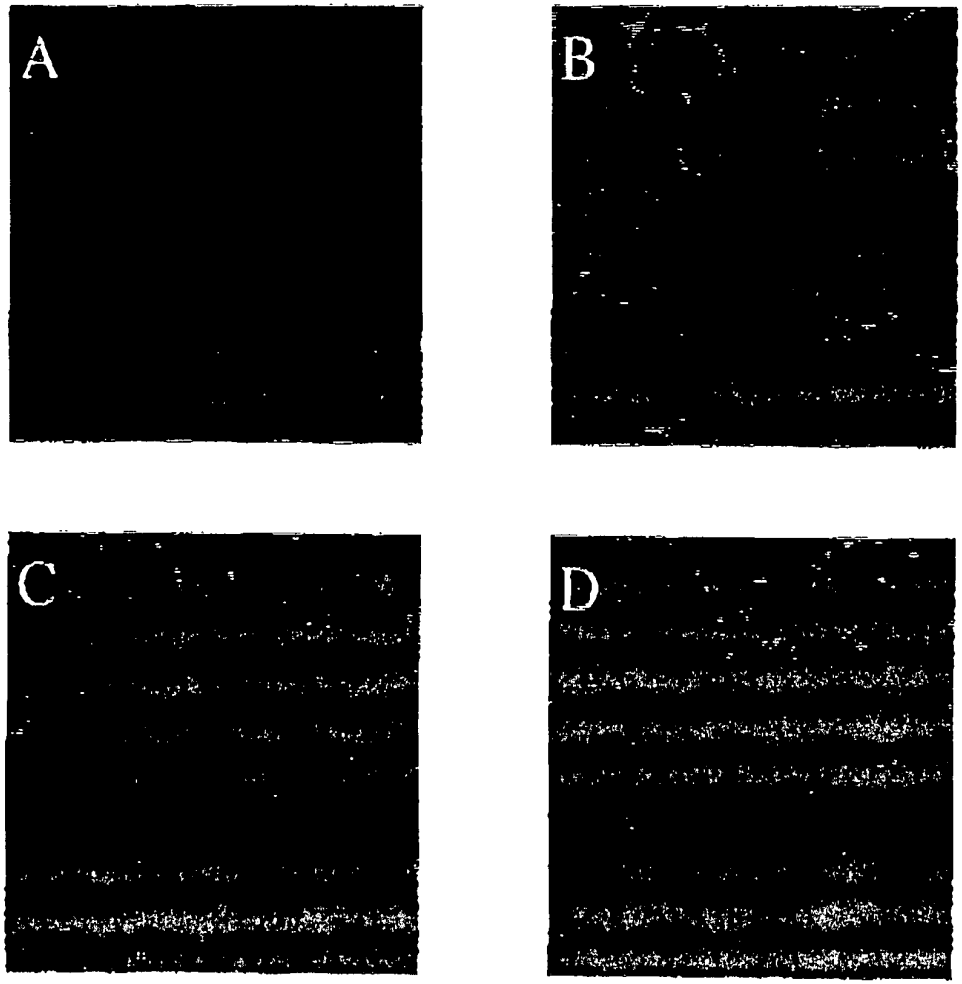


FIGURE 6a



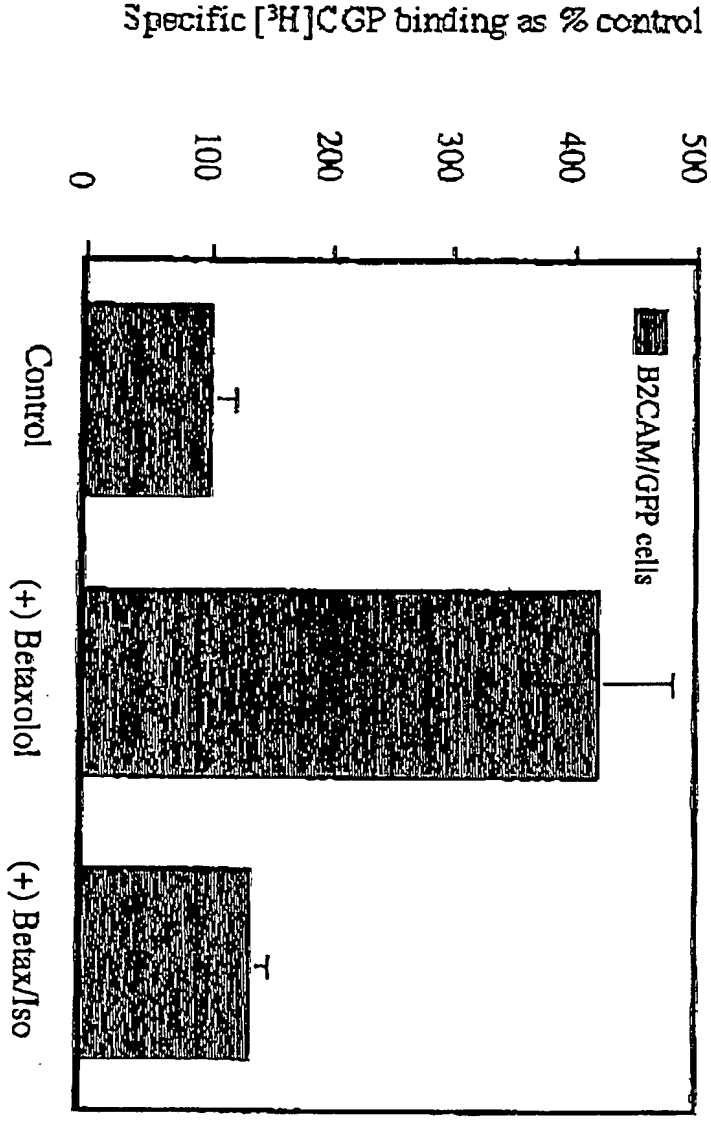

Specific [³H]CGP binding as % control
0
100
200
300
400
500
B2CAM/GPP cells
Control
(+) Betaxolol
(+) Betax/Iso

FIGURE 6b

FIGURE 7a

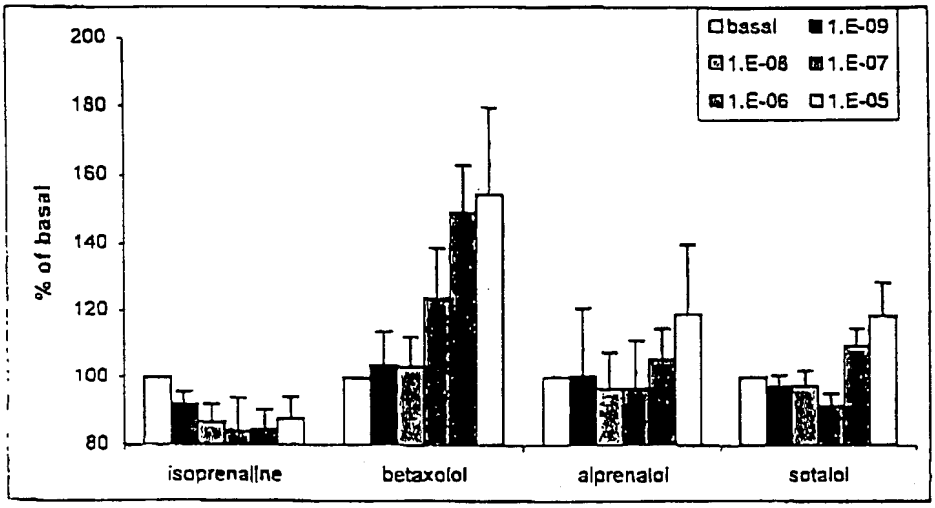

basal
1.E-09
1.E-08
1.E-07
1.E-06
1.E-05
% of basal
200
180
160
140
120
100
80
isoprenaline
betaxolol
alprenalol
sotalol

FIGURE 7b

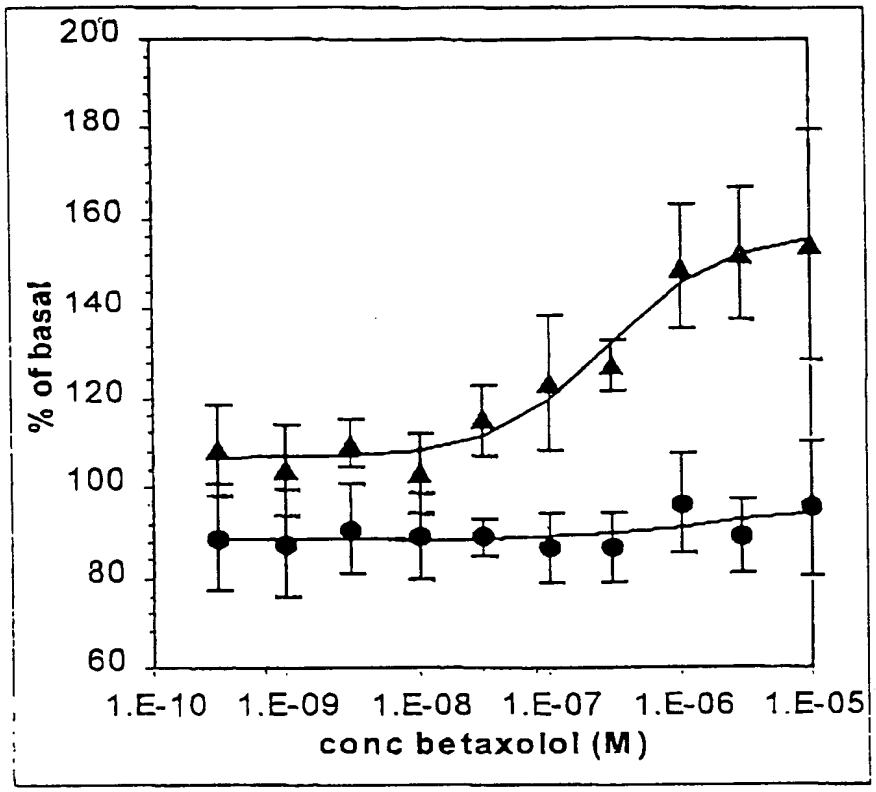

200
180
160
140
120
100
80
60
% of basal
1.E-10
1.E-09
1.E-08
1.E-07
1.E-06
1.E-05
conc betaxolol (M)

FIGURE 7c

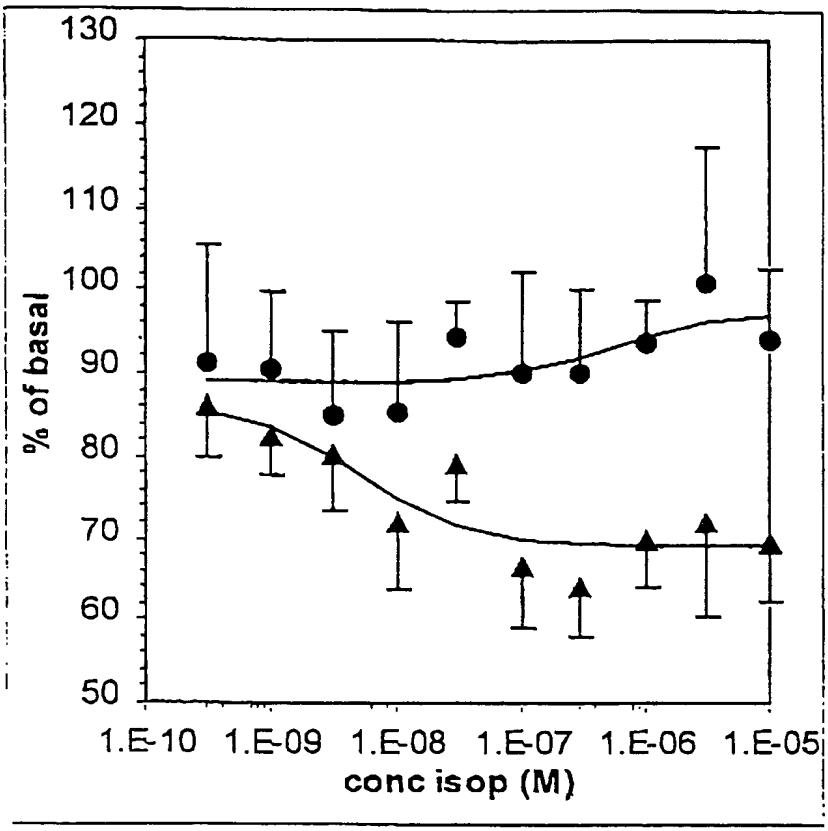
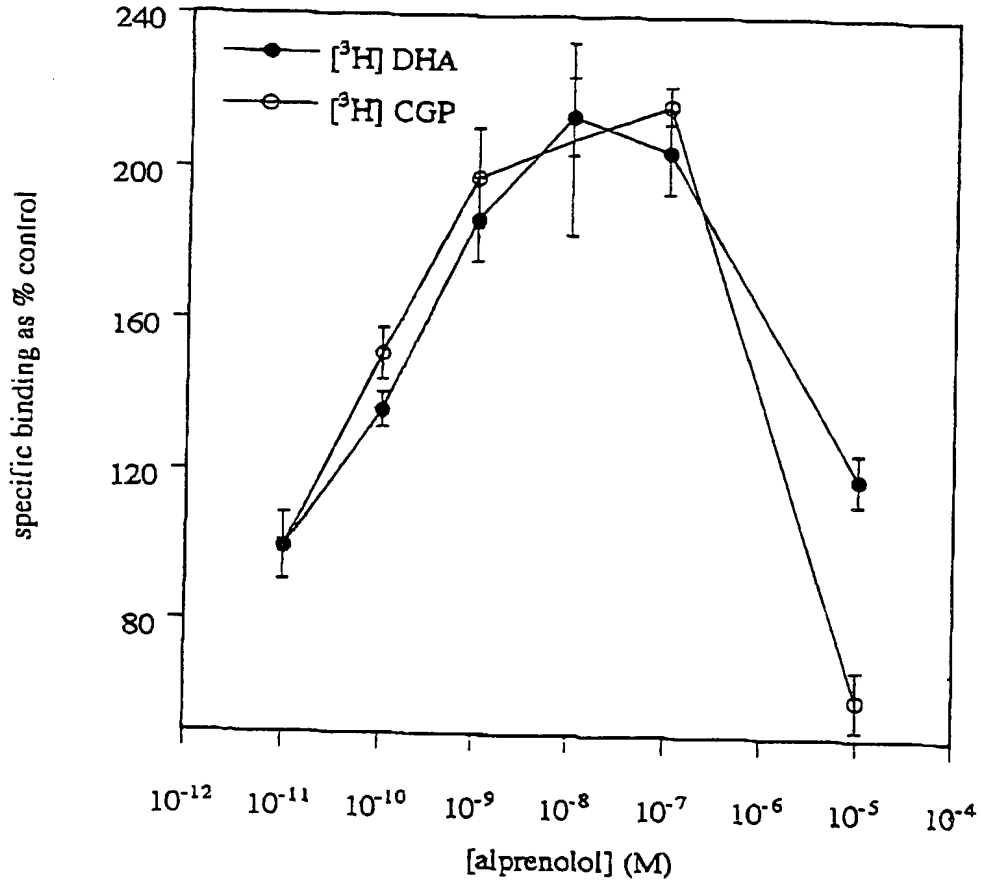

130
120
110
100
90
80
70
60
50
% of basal
1.E-10
1.E-09
1.E-08
1.E-07
1.E-06
1.E-05
conc isop (M)

FIGURE 8a


240
200
160
120
80
[^{3}H] DHA
[^{3}H] CGP
specific binding as % control
10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
[alprenolol] (M)